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1 Attachment

Use of reflectance spectrophotometry-Hughs & Langlois.pdf

Dear Judge Browning and Counsel:

Per AUSA's Neda and Peña, attached please find the final medical journal article.
Josh Ruden
Paralegal Specialist
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District of New Mexico
505-224-1539

From: Ruden, Joshua E. (USANM)
Sent: Wednesday, September 17, 2014 4:51 PM
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Cc: K'Aun_Wild@nmcourt.fed.us; Neda, Tara (USANM); Pena, Jeremy (USANM) 2
Subject: U.S. v. Rodella 14cr2783 JB-Medical Journal Articles

Dear Judge Browning and Counsel:

Per AUSA's Neda and Peña, attached please find four of the five requested medical journal articles. The last article should be forthcoming sometime tomorrow morning, depending on when it's emailed to this office.

Josh Ruden
Paralegal Specialist
U.S. Attorney's Office
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<< File: Estimation of the age of bruising-Stephenson & Bialas.pdf >> << File: Forensic Sci Int.pdf >> << File: Journal of Forensic and Legal Medicine.pdf >> << File: Medicine Science and the Law.pdf >>

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Use of reflectance spectrophotometry and colorimetry in a general linear model for the determination of the age of bruises

Vanessa K. Hughes · Neil E. I. Langlois

Abstract Bruises can have medicolegal significance such that the age of a bruise may be an important issue. This study sought to determine if colorimetry or reflectance spectrophotometry could be employed to objectively estimate the age of bruises. Based on a previously described method, reflectance spectrophotometric scans were obtained from bruises using a Cary 100 Bio spectrophotometer fitted with a fibre-optic reflectance probe. Measurements were taken from the bruise and a control area. Software was used to calculate the first derivative at 490 and 480 nm; the proportion of oxygenated hemoglobin was calculated using an isobestic point method and a software application converted the scan data into colorimetry data. In addition, data on factors that might be associated with the determination of the age of a bruise: subject age, subject sex, degree of trauma, bruise size, skin color, body build, and depth of bruise were recorded. From 147 subjects, 233 reflectance spectrophotometry scans were obtained for analysis. The age of the bruises ranged from 0.5 to 231.5 h. A General Linear Model analysis method was used. This revealed that colorimetric measurement of the yellowness of a bruise accounted for 13% of the bruise age. By incorporation of the other recorded data (as above), yellowness could predict up to 32% of the age of a bruise— implying that 68% of the variation was dependent on other factors. However, critical appraisal of the model revealed that the colorimetry method of determining the age of a bruise was affected by skin tone and required a measure of the proportion of oxygenated hemoglobin, which is obtained by spectrophotometric methods. Using spectrophotometry, the first derivative at 490 nm alone accounted for 18% of the bruise age estimate. When additional factors (subject sex, bruise depth and oxygenation of hemoglobin) were included in the General Linear Model this increased to 31%—implying that 69% of the variation was dependent on other factors. This indicates that spectrophotometry would be of more use that colorimetry for assessing the age of bruises, but the spectrophotometric method used needs to be refined to provide useful data regarding the estimated age of a bruise. Such refinements might include the use of multiple readings or utilizing a comprehensive mathematical model of the optics of skin.

Keywords Bruise · Spectrophotometry · Colorimetry · Time factors

Introduction

The bruise is a significant injury in the eyes of the law in Australia. A bruise results from blunt force injury to the skin when the force is sufficient to rupture blood vessels within the skin, resulting in extravasation of blood and the formation of a bruise or skin hematoma [1, 2]. Although the color of blood is red, due to the hemoglobin molecule, the appearance of extravasated blood within the skin varies according its oxygenation and the depth of its location below the skin's surface [3–6]. When blood is released into the skin as a result of trauma, there is an ensuing inflammatory reaction: hemoglobin is catabolized into the yellow pigment bilirubin [7, 8] and the golden-brown pigment hemosiderin. Eventually, bilirubin and hemosiderin are

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removed by the macrophages and a healing reaction follows with a gradual fading of color.

An estimation of the age of a bruise may be attempted by direct visual examination or by the inspection of photographs taken of the injured area [1, 9, 10], but these methods are subjective in nature [11, 12] and may not be reliable. Forensic investigators would benefit from a non-invasive, objective, scientifically validated method for determining the age of bruises. Previous authors have suggested that either colorimetry or reflectance spectrophotometry might provide a solution [13–15].

Colorimetry can be used to measure the color of a surface. Many different systems have been developed to numerically express the visual perception of color. The Commission Internationale de l'Éclairage (CIE) proposed a method of standardized color measurement, the CIE-$L^*a^*b^*$ system, which takes into account the non-linear color perception of the human eye [16, 17]. In the CIE-$L^*a^*b^*$ system, the L^* value defines the relative brightness (or luminosity) of the sample ranging from total black ($L^* = 0$) to total white ($L^* = 100$). The a^* value is the red-green coordinate. A positive a^* value defines the color quality "red" and a negative a^* value defines the color quality "green". The b^* value is the blue-yellow coordinate. A positive b^* value defines the color quality "yellow" and a negative b^* defines the color quality "blue" [17]. A colorimeter can be used to calculate the relative luminosity and hues of a surface, such as skin, thus allowing a more accurate, non-subjective assessment of color than by visual assessment [18, 19]. Colorimetry has been used to evaluate skin color and postmortem hypostasis [15, 20–23]. For the purpose of this study, yellowness was measured using the calculated b^* value and by using two specific measures of yellow: the American Society for Testing Materials (ASTM) E313 and D1925 values [24].

Reflectance spectrophotometry measures the change in light intensity relative to its wavelength after it has interacted with a sample. The device comprises a light source, a probe and an analyzer, and the results are recorded on a dedicated computer. The spectrophotometer measures the intensity of light throughout the spectrum and is an accurate and objective device [25]. Over the range 470–510 nm the absorption spectrum of hemoglobin is nearly flat, but the absorption of bilirubin is decreasing from its peak around 460 nm [26, 27] and hemosiderin has a sloping absorption curve [28]. Therefore, the value of the first derivative around 480–490 nm corresponds to the presence of degradation products of hemoglobin [25]. When blood is released into the tissue, it would be expected to be predominantly in the form oxyhemoglobin which would relinquish its oxygen in the tissue to become predominantly deoxyhemoglobin [14]. The ratio of oxyhemoglobin to deoxyhemoglobin can be calculated using differences in the absorption spectra. To date, investigators using spectrophotometry have studied only small groups [29–31].

This study aims to determine if reflectance spectrophotometry or colorimetry can be used to determine the age of a bruise, using a large group of subjects with bruises of known age.

Materials and methods

Reflectance spectra were collected from normal skin and bruises in 149 volunteers using a Cary 100 Bio UV–visible spectrophotometer fitted with a Cary fiber optic couple and Cary fiber optic reflectance probe (Varian Australia Pty Ltd., Frenches Forrest, New South Wales, Australia). The instrument was calibrated before each series of measurement using zero and baseline corrections as previously described [25]. Calibrations and measurements were performed under similar conditions. Subjects were measured in a seated position and were asked to remain still for 5 min before any readings were taken. All measurements were taken from the central region of the bruise. An area of non-bruised skin adjacent to the bruised area was also measured and used as the control. Subjects were instructed to hold the probe gently and completely perpendicular to the skin so as to prevent light from entering the probe or pressure from affecting the measurements obtained. To avoid skin color changes due to excessive contact or friction against the probe heads, only one measurement was made on the test site, as has been recommended [22]. The absorption spectrum of the bruise was obtained over the range 830–360 nm. A data interval of 1 nm was selected, with a signal averaging time of 0.2 s for each data point (resulting in a scan time of 70 s). Double beam mode was used with 2.5 Abs of rear beam attenuation and the slit width was set to 3.0 nm.

The volunteers worked at or had been admitted as patients to Westmead Hospital or the Children's Hospital, NSW, Australia, and the age, sex, skin color and approximate body size of each volunteer were recorded. The extent of trauma, age of the bruise, and the site of the bruise were also recorded for each subject (see below). The age of the bruise at the first and subsequent consultations was also recorded. Some subjects presented with new bruises on multiple occasions, other subjects came for repeat examination of the same bruise. Subjects were not examined more than once in a 24 h period. Ethics permission was obtained from both the Western Sydney Area Human Research Ethics Committee [HS/TG HREC2002/5/4.6(1432)] and ratified by the Human Research Ethics committee at the University of Sydney [3231] before subjects were recruited. To be eligible to participate in this study, subjects were required to know the age and cause of

the bruise that was to be examined, be in good health, have no difficulty conversing in English and able to give written consent. All subjects were aged 18 years or older. Only bruises on legs and arms that were easy to access while the subject remained clothed were used. Subjects with cuts or abrasions on the surface or near the bruise were also excluded, to prevent potential contamination of the wound or probe. Volunteers with excessive hair over the bruised region were also excluded as hair was found to interfere with measurements. All volunteers recruited for the study had bruises that had been acquired from recreational or normal daily activities. No identifying data were collected.

The age and gender of each subject was recorded. The subject's body size and build was visually assessed. Using this assessment subjects were classified into three body size classes: 'Underweight'—very little fat content, prominent bones and muscles, 'Normal weight'—normal fat for body size, and 'Overweight'—increased fat for body size. The bruise size was measured and the subject was asked how it had been acquired. Using this information the bruise was classified as being either (1) a small bruise caused by a minor trauma, (2) a large bruise caused by a minor trauma, (3) a small bruise caused by a major trauma, (4) or a large bruise caused by a large trauma. Minor trauma was be defined as the type of trauma a person would experience if he or she walked into an object, e.g., a coffee table. Major trauma was defined as the type of trauma experienced if hit by a car or by a paintball. The bruise was regarded as 'small' if it was less than 5 cm in diameter and 'large' if was 5 cm or larger. The skin tone of each subject was determined by measuring the CIE$L*a*b*$ "$b*$" value of an area of adjacent control skin and was classified as: $b*$ +0.10 to 0.5 'Fair', $b*$ −0.51 to −1.50 'Medium', b −1.51 to −3.0 'Olive to Dark'. The location of the bruise was coded into one of four categories: upper limb, hand, lower limb, foot.

All bruises were photographed. These were examined by a forensic pathologist (NL) who classified them as being intradermal, deep (subcutaneous) or mixed using the criteria of Bohnert [3].

The oxygenation of hemoglobin within the bruise was calculated by subtracting the absorption at an isosbestic point (805 nm) and a nonisosbestic point (660 nm). The formula {Bruise 805 nm/Bruise 660} − {Control 805 nm/ Control 660} was used to measure the oxygenation of hemoglobin, which is an adaptation of the method proposed by Randenburg et al. [31].

The first derivative was calculated using the mathematical software provided with the Cary Win UV spectrophotometer. The native trace of both the control site and the bruised site were converted to the first derivative using a filter size of 9 and a bandwidth of 3. The values obtained at 480 and 490 nm in the control value was subtracted from the value obtained at 480 and 490 nm in the bruise scan, respectively.

Scans were converted into CIE$L*a*b*$ values using the Cary Win UV color application, 85-101684-00 version 2.00(15) supplied by Star-Tek (Victoria, Australia). The CIE$L*a*b*$ values were calculated based over a scan range of 830–360 nm using a 1 nm data interval. The results were based on a CIE D65 illuminant with an observer angle of two degrees for the CIE$L*a*b*$ color space. After testing the software using a Kodak color card (results not shown) it was found that the software produced a negative $b*$ value to indicate yellowness. Therefore, in order for our results to truly reflect the CIE$L*a*b*$ system and prevent confusion, the signs of all results were changed to the corresponding positive value. The value obtained for the CIE$L*a*b*$ "$b*$" value from the control scan was also used to determine the color of the subject's skin.

Statistical analysis was performed using SPSS Student Version 11 (SPSS Inc, Prentice Hall, UK). The possible confounding subject-related variables were analysed using Spearman's correlation to determine if there was any correlation between them and the age of a bruise. A general linear model univariate analysis was then conducted using the reflectance spectrophotometry and colorimetry methods that had a moderate correlation with the age of a bruise and the possible confounding variables, so as to determine if it was possible to create a model that could predict the age of a bruise. Using a backward stepwise selection process, variables were removed until only the significant variables remained.

All measurements of the first derivatives at 480 and 490 nm as well as the color measurements $b*$, E313 and D1925 used in the analyses refer to the bruise minus the control value.

Results

A total of 147 subjects were recruited for this study comprising 28 males and 119 females with ages ranging from 18 to 72 years (mean = 35.88, median = 32.00, SD = ±11.47). A total of 240 scans were obtained, of which 233 were of sufficient quality to allow analysis. The age of the bruise at the first and subsequent consultations was recorded; the ages of the bruises ranged from 0.5 to 231.5 h (mean = 76.39 h, median = 72.00 h, SD = ±48.56 h). The frequency distribution of the scans acquired for bruises of different age groups is shown in Fig. 1; the frequencies of subjects and scans made for categories of body build, bruise size and degree of trauma, bruise site, and depth of bruise is shown in Table 1.

The results of the general linear model analysis indicated that measurement of yellow using the CIE $b*$ value
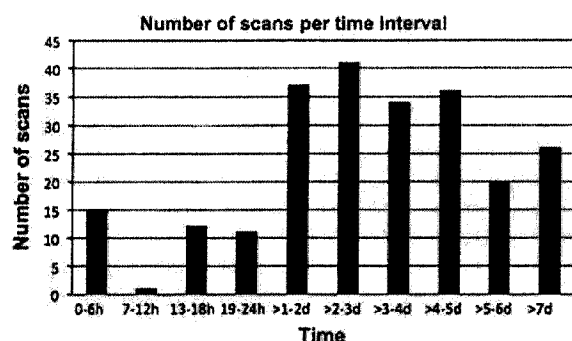
Fig. 1 Number of scans of bruises in time intervals (note non-linear X-axis)

Table 1 Frequencies of subjects and scans made for categories of body build, bruise size and degree of trauma, bruise site, and depth of bruise

	Number of subjects	Number of scans
Frequency of body build of volunteers		
Build		
Slender	9	16
Average	92	141
Large	46	76
Total	147	233
Frequency of bruise size and trauma sustained		
Bruise size/trauma		
Small bruise/minor trauma	111	177
Large bruise/minor trauma	22	32
Small bruise/major trauma	12	19
Large bruise/major trauma	2	5
Total	147	233
Frequency of site of bruise		
Site		
Arm	60	98
Hand	22	28
Leg	58	97
Foot	7	10
Total	147	233
Frequency of depth of bruise		
Depth		
Deep	114	178
Intra-dermal	5	7
Mixed	28	48
Total	147	233
Number of volunteer in each skin tone catagory		
Skin tone		
Fair	67	121
Medium	60	85
Olive/dark	20	27
Total	147	233

by colorimetry accounted for only around 12% of the predicted age of the bruise. The use of the specific measures of yellowness, E313 and D1925, improved the prediction to 13%. The best estimate of bruise age was obtained using the calculated ASTM E313 value by adding the gender of the subject, an estimate of the depth of the bruise, the spectrophotometric measurement of the proportion of oxygenated hemoglobin, the site of the bruise and skin tone into the analysis. However, the ASTM E313 value in conjunction with these factors only predicted 32% of the age of the bruise, meaning that 68% of the variation between the observed and predicted bruise age was dependent on other factors.

The calculation of the first derivative values showed similar results, with the first derivative at 490 nm being slightly superior to the value at 480 nm (alone estimating 18 and 14% of the bruise age, respectively). The general linear model indicted that only the gender of the subject, the estimated depth of the bruise, and the calculation of the proportion of oxygenated hemoglobin improved the ability of the first derivative method to predict the age of bruises. Using these additional factors first derivative at 490 nm accounted for at best 31% of the predicted age of the bruise, leaving 69% of the prediction of the age of bruise to other factors.

The measurements of yellow (using b^*, E313 and D1925) as well as the values of the first derivative calculations (at 480 and 490 nm) were plotted against the known age of the bruise. These plots were examined to assess the changes at the very early times of 0.5–24 h. There was no clear trend to indicate if accumulation of degradation products of hemoglobin or if the development of yellow color occurred gradually from time zero, or if there was a lag. Overall for all the data, the best curve of fit was a simple parabola, showing an increase to around 7 days and then a decline (Fig. 2). The first derivative at 490 nm measures the amount of bilirubin and hemosiderin while the ASTM E313 color number measures the amount of yellow. The correlation between the two values was strong with a correlation of coefficient squared (r^2) value of 0.789 ($P \geq 0.001$). The yellowness ASTM E313 color number was divided by the first derivative at 490 nm and plotted against the age of the bruise; most of the plotted points lay along a straight, horizontal line as would be expected (Fig. 3). However, there was a degree of scatter indicating that measured yellow color did not always relate to the presence of hemoglobin breakdown products.

Discussion

This study has confirmed that observation of the color yellow in a bruise or the measurement of the presence of
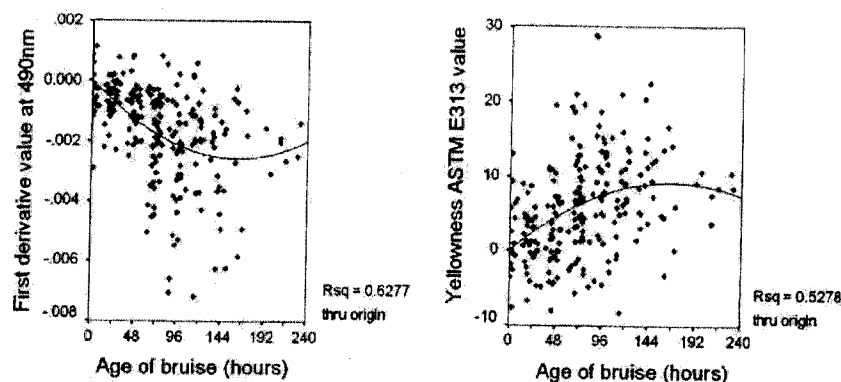
Fig. 2 Time trends of first derivative at 490 nm (*left*) and yellowness ASTM E313 (*right*) with line of best fit

breakdown products of hemoglobin do provide information regarding the age of a bruise. However, by themselves these are of limited use. Using additional factors increases the ability to predict the age of bruises, but the predictive values must be regarded as too low to be useful, providing around only one-third of the age estimation using the general linear model with the methodology of this study.

The factors that improve the accuracy of the bruise age prediction using colorimetry include the proportion of oxygenated hemoglobin, the site of the bruise and skin tone. As the proportion of oxygenated hemoglobin within a bruise is derived using spectrophotometry, it is not applicable to measurements of yellow color made using only a colorimeter which uses a light source and three receptors from which color data is calculated [22, 32]. Skin tone should not have been incorporated into the multivariate general linear model as the yellow measurement of control skin had been subtracted from the bruise color measurement. The result indicated that subtraction of the control was not working adequately. This may also account for some of the lack of correlation between the measurement of yellowness and the first derivative value and the scatter seen in Fig. 3. The finding also accounts for the inclusion of the site into the multivariate general linear model for colorimetric prediction of bruise age. Critical analysis revealed that the hand was the only site that was significantly different (out of hand, arm, leg and foot that were coded) and that it was consistently appearing more yellow at a given time than expected. When a bruise on the hand was recorded, the control site was usually taken proximally, further up the extremity. Observation revealed that a person's hand tends to be more yellow (a finding also recorded by others [33]), presumably due to sun exposure, than the arm. Thus, due to imperfect subtraction by the control, bruises of the hand were appearing more yellow at a given time than bruises on other sites.

Although it appeared that colorimetry provided the best prediction of bruise age when combined with additional factors, critical analysis shows colorimetry to be impaired by the underlying skin color.

Measurement of the first derivative values was not affected by skin tone in the study group. However, there were no deeply pigmented, negroid, subjects. The addition of subject sex as a significant factor was not expected, with females displaying more breakdown of hemoglobin at a given age of bruise than males. However, the small number of male subjects questions the validity of this observation. The depth of the bruise as judged by inspection was also a significant variable. This was not unexpected. However, the fact that mixed depth bruises showed more breakdown of hemoglobin at a given age of bruise than deep intradermal, coupled with the low number of purely intradermal bruises places uncertainty on this finding.

It has been written that a bruise will not appear yellow in under 18 h [1]. This observation has been supported by others. For yellow to be seen in a bruise the local accumulation of yellow pigments of hemoglobin breakdown needs to exceed the background color of skin by a sufficient level to be perceived by the human visual system. The requirement for a period of time before the appearance of yellow color could be due to a true delay resulting from the need to recruit macrophages to the injured area and then for macrophages to induce their heme oxygenase enzymes to allow the degradation of hemoglobin. It was hoped to be able to demonstrate a lag phase in the development of yellow color and the change in the first derivative values, however, this was not clearly present (Fig. 2). A lack of data from early bruises may be partly the reason for this (Fig. 1) and for the colorimetry data: the inability to completely correct for skin tone may have caused obfuscation.

This large spectrophotometry study of bruises has demonstrated that there is a relationship between the
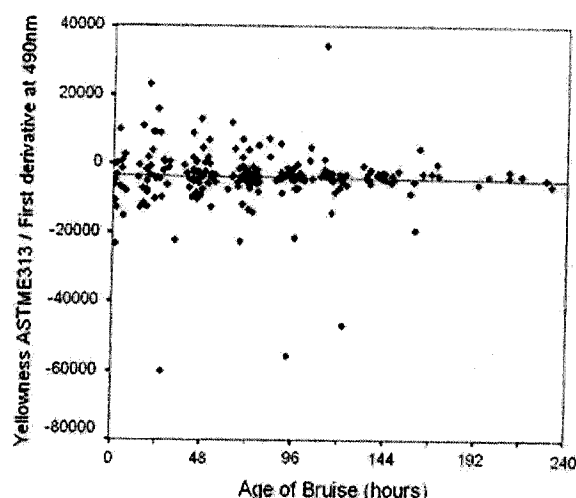
Fig. 3 Yellowness ASTM E313 divided by first derivative at 490 nm against time. Although there is good correlation between the two variables ($r^2 = 0.8$), it can be seen there are a number of outlier points

demonstration of breakdown products of hemoglobin and the age of a bruise. However, the relationship is a weak one and other factors have to be taken into account. Colorimetry measurements can be significantly affected by skin tone. For spectrophotometry, subject dependent factors include gender and bruise depth. The size of the bruise, the degree of trauma, subject build, bruise site and subject age were not factors that influenced being able to determine the age of the bruise.

This study is limited by the small numbers in some subject groups (for example the number of male subjects, the number of purely intradermal bruises and small numbers of early bruises). Also, only a single measurement was taken from each bruise using the probe which has a sampling area around 3 mm in diameter. The results suggest that simple analysis of spectrophotometric data of this type is of limited use in determining the age of bruises. The use of complex mathematical modeling based on the optical properties of skin combined with collection of spectrophotometric data over larger areas has shown promise in bruise age determination [31], however, in the published study the subjects and bruises were similar. It is possible that subject related variables will need to be included into any methodology that is used to determine the age of bruises by spectrophotometry.

Key points

1. Measurement of yellow color using colorimetry provides information regarding the age of a bruise. However, measurements appear affected by skin tone.

2. Measurement of the accumulation of the degradation products of hemoglobin by using reflectance spectrophotometry to obtain the first derivative value at 490 nm provides information regarding the age of a bruise, which is not affected by skin tone.

3. Subject related factors such as gender, depth of the bruise and spectrophotometric measurement of the oxygenation of hemoglobin in the bruise increase the predictive value of the first derivative value at 490 nm.

4. Even when subject related factors are incorporated into the general linear model with first derivative value at 490 nm, using the methodology of this study provides only 31% of the prediction of the age of a bruise.

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